Effect of Salinity on Nodule Formation by Soybean[1]

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ABSTRACT

A split-root growth system was employed to evaluate the effect of NaCl on nodule formation by soybean (*Glycine max* L. Merr. cv Davis). By applying the salinity stress and rhizobial inoculum to only one-half the root system, the effects of salinity on shoot growth were eliminated in the nodulation process. *Rhizobium* colonization of inoculated root surfaces was not affected by the salt treatments (0.0, 26.6, 53.2, and 79.9 millimolar NaCl). While shoot dry weight remained unaffected by the treatments, total shoot N declined from 1.26 grams N per pot at 0.0 millimolar NaCl to 0.44 grams N per pot at 79.9 millimolar NaCl. The concentration of N in the shoot decreased from 3.75% N (0.0 millimolar NaCl) to 1.26% N at 79.9 millimolar NaCl. The decrease in shoot N was attributed to a sharp reduction in nodule number and dry weight. Nodule number and weight were reduced by approximately 50% at 26.6 millimolar NaCl, and by more than 90% at 53.2 and 79.9 millimolar NaCl. Nodule development, as evidenced by the average weight of a nodule, was not as greatly affected by salt as was nodule number. Total nitrogenase activity (C_2H_2 reduction) decreased proportionally in relation to nodule number and dry weight. Specific nitrogenase activity, however, was less affected by salinity and was not depressed significantly until 79.9 millimolar NaCl. In a second experiment, isolates of *Rhizobium japonicum* from nodules formed at 79.9 millimolar NaCl did not increase nodulation of roots under salt stress compared to nodule isolates from normal media (0.0 millimolar NaCl). Salt was applied (53.2 millimolar NaCl) to half root systems at 0, 4, 12, and 96 hours from inoculation in a third experiment. By delaying the application of salt for 12 hours, an increase in nodule number, nodule weight, and shoot N was observed. Nodule formation in the 12- and 96-hour treatments was, however, lower than the control. The early steps in nodule initiation are, therefore, extremely sensitive to even low concentrations of NaCl. The sensitivity is not related to rhizobial survival and is probably due to the salt sensitivity of root infection sites.

Successful initiation of nodulation and nitrogen fixation by a genetically compatible legume-*Rhizobium* combination has two prerequisites: (a) colonization of root surfaces and attachment of rhizobia to roots; (b) infection of root hairs. Stress factors such as soil salinity may have an adverse effect on these two processes and limit nitrogen fixation by reducing nodule number.

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Rhizobium growth and survival are generally more tolerant *in vitro* to high osmotic pressures than are their respective host legumes (8, 15, 17). Tu (22), however, observed reduced colonization of soybean root surfaces by *Rhizobium japonicum* when plants were grown in salinized culture medium.

Legumes grown in saline environments exhibit reduced yield potential and reduced numbers and weight of root nodules (1, 14, 15, 22, 24). There were, however, serious limitations in the above studies for evaluating the effects of salinity on the early stages of nodule formation. With the exception of the work of Lakshmi *et al.* (14), inoculation of seedlings with *Rhizobium* preceded the salinization of the rooting medium. It is likely that in these studies some critical steps of rhizobial attachment and infection thread formation could have occurred before the introduction of the salt stress. In the study of Lakshmi *et al.*, (14) plant growth was so restricted that even nonsalinized control plants of *Medicago sativa* had less than two nodules per plant.

Previous work concerning the effects of salinity on nodule initiation also suffers from the fact that plant yield potential was affected by the salinity treatments. Reduced shoot growth, resulting even from non-soil related stress such as low light intensity, also reduces nodule number (21). This relationship between shoot yield potential and nodule initiation requires, therefore, that the stress imposed upon the site of nodule initiation does not affect shoot growth.

In this paper, the sensitivities of rhizobial colonization of root surfaces and nodule initiation to salinity are examined. Plant growth potential as a confounding variable was eliminated by employing a split-root growth system described by Singleton (18). This system permitted the application of increasing salt concentrations to nodule initiation sites without affecting plant growth potential.

MATERIALS AND METHODS

Plant Culture. Eight soybean seeds (*Glycine max* L. Merr. cv Davis) were planted in a split-root solution culture growth system (19 L capacity on each side) as described by Singleton (18). The experiment was conducted in a glasshouse during December 1981 and January 1982. The nutrient solution consisted of 0.5 mM P, 0.96 mM K, 1.56 mM S, 0.82 mM Mg, and 0.75 mM Ca. Sources were: K_2HPO_4; $MgSO_4 \cdot 7H_2O$; $CaSO_4$.

Micronutrients were added according to Broughton and Dilworth (7). Nitrogen (3.6 mM) as NH_4NO_3 was added to each container at planting. Eighteen days after planting solutions were replaced with N-free nutrient solution. NaCl was added to one container of the split-root assembly at concentrations of 0.0, 26.6, 53.2, or 79.9 mM NaCl. Two hours later, the salinized side was inoculated with enough *Rhizobium japonicum* strain USDA 110 to produce a viable cell density of 1.2×10^6 cells/ml plant nutrient solution. Two days later, 0.7 mM N as NH_4NO_3 was added to the uninoculated side to maintain leaf area and vigorous plant growth during the early stages of nodule formation. Solutions were sampled for enumeration of *Rhizobium* and changed at 46 d from planting. Reinoculation of salinized half-root sys-

tems was at 9.9×10^4 cells/ml plant nutrient solution. The four treatments were replicated three times in a completely randomized design. Water uptake was monitored daily through a calibrated sight glass in each container and solutions were replenished when the level fell by 2 L.

Harvest. Shoots and roots were cut 54 d after planting. Roots were incubated in 5.0% (v:v) acetylene in 2.0-L plastic containers for 30 min. Ethylene production was determined by GC. Nodules and roots were subsampled for reisolation and the *Rhizobium* colonization study and stored at 4°C. The remaining nodules were removed from roots and nodules, roots, and shoots were dried at 65°C. Shoot N was determined by micro-Kjeldahl.

Rhizobium Culture. Yeast extract mannitol broth cultures were counted by the drop plate method (23) and then centrifuged at 12,100g and 4°C. Cells were resuspended in water for inoculation.

Enumeration of *Rhizobium* in Plant Nutrient Solution. One-ml aliquots of nutrient solution samples were diluted in distilled H_2O and filtered through a 0.4-μm Nucleopore polycarbonate membrane filter that had been stained with Irgalan Black. Filters were treated with USDA 110 fluorescent antibody prepared according to the methods of Schmidt *et al.* (16). Cell counts were made by fluorescence microscopy.

Colonization of Root Surfaces by *Rhizobium*. Root sections (2 cm) that had been stored at 4°C were incubated for 30 min in an Eriochrome Black solution prepared according to the method of Goldman (10) with the exception that dimethylsulfoxide was substituted for *N,N*-dimethylformamide. Roots were rinsed in water until all excess dye was removed, then treated with gelatin-rhodamine isothiocyanate (6). Root sections were then incubated with USDA 110 fluorescent antibody for 30 min. Random microscope fields (100) were examined for positive antibody reaction. A field with any fluorescent cells was counted as being colonized.

Isolation and Testing of *R. japonicum* Strain USDA 110 from Nodules Formed in Highly Salinized Rooting Medium. Isolates were made from the unsalinized controls and from the few nodules formed when the nodule initiation process was exposed to 79.9 mM NaCl. Isolates were identified as being strain USDA 110 by immunofluorescence microscopy (16). Two isolates from unsalinized controls and two from the 79.9 mM NaCl treatment were then inoculated to half-root systems with either 0.0 mM NaCl or 79.9 mM NaCl in the rooting medium. The plant growth system has been described earlier. Inoculation was at 20 d from planting. Nutrient solutions were changed prior to inoculation, and NaCl at 79.9 mM was added to designated half-root systems 2 h prior to inoculation. Cell densities were 9×10^5/ml nutrient solution for both strains. Nitrogen (0.35 mM) as NH_4NO_3 was added to the uninoculated side at 23 and 27 d from planting. Harvest was 52 d after planting. The experiment was conducted in a glasshouse during February and March 1982.

Effect of Time of Salt Addition on Nodule Formation. A third experiment was conducted to examine the interaction between time of salt treatment initiation, inoculation, and nodule formation. The planting and growth system methodologies were as before except that, at inoculation (5 × 10⁵ cells/ml nutrient solution) 18 d from planting, a time course for the addition of NaCl to inoculated half-root systems was begun. Salt (53.2 mM NaCl) was added to inoculated half-root systems at either 0, 4, 12, or 96 h from the time of inoculation. Controls had no salt added to the inoculated half-root system. The treatments were replicated twice and are presented without statistical analysis. The experiment was conducted in a glasshouse during August and September 1982.

RESULTS

There was no treatment effect on the fluorescent antibody counts of *R. japonicum* strain USDA 110 in the nitrogen-free

Table I. *Effect of NaCl in the Rooting Medium on Survival of Rhizobium japonicum Strain USDA 110 and Colonization of Soybean Roots*

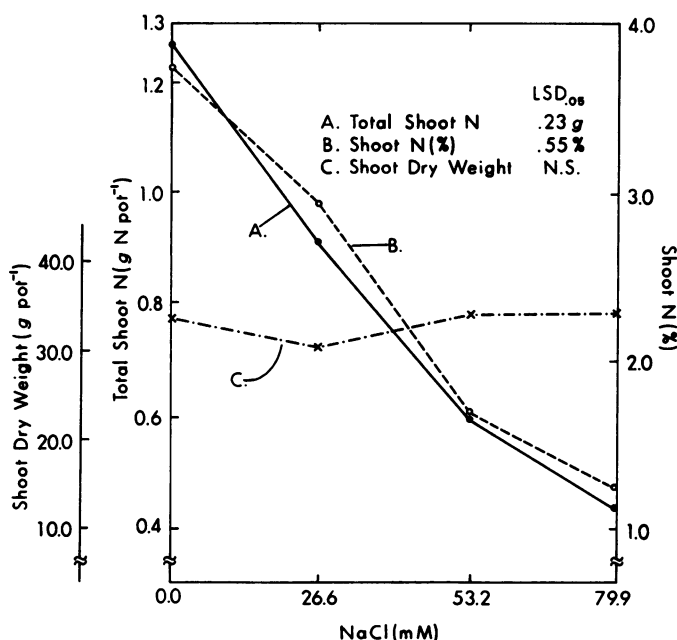| NaCl	No. of Cells in Rooting Medium	Fields Colonized
mM	*log no. ml⁻¹*	%
0.0	4.11	92
26.6	3.96	76
53.2	4.36	82
79.9	4.02	91
LSD 0.05	NS	

FIG. 1. Effect of NaCl applied 2 h prior to inoculation on shoot dry weight, shoot N, and the concentration of N in the shoot.

nutrient solution. Colonization of roots was similarly not affected by salinization of the rooting medium (Table I).

Total shoot N and concentration of N in the shoot declined as the concentration of NaCl applied to the half-root system at inoculation increased. Shoot weight was not affected by the salinity treatments (Fig. 1).

Exposing the infection process to NaCl resulted in a sharp reduction in nodule number, nodule mass, and total nitrogenase activity (Fig. 2). Specific nitrogenase activity was more resistant to salt stress; a significant reduction was not evident except at the highest level of salt employed (79.9 mM NaCl).

Exposure of a half-root system to increasing concentrations of NaCl reduced root growth by that side which was compensated for by increased root proliferation on the nonsalinized side. Water uptake by the two sides followed a similar trend (Fig. 3).

Delaying the application of NaCl to an inoculated half-root system resulted in an increase in nodule number, nodule mass, and total shoot N compared to salinization at inoculation (Table II).

Isolates made from root-nodules that developed in the 0.0 and 79.9 mM NaCl treatments in the first experiment were not different in their ability to nodulate the host with 79.9 mM NaCl in the rooting medium (Table III).
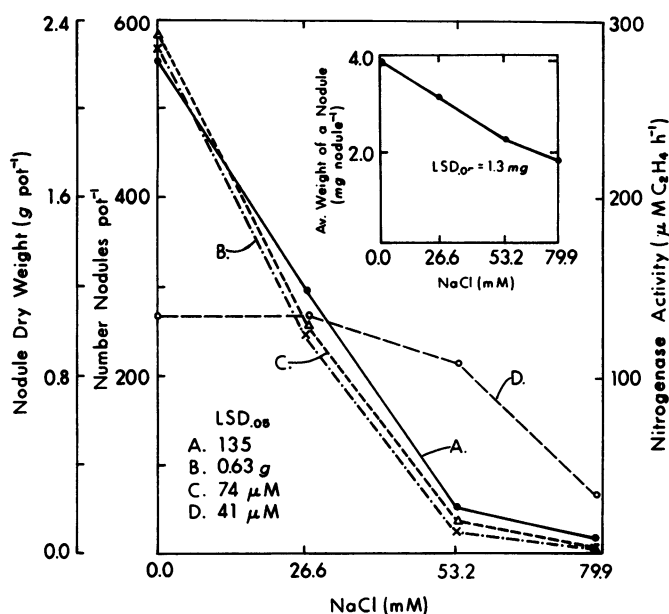
FIG. 2. Effect of NaCl applied 2 h prior to inoculation on nodule number (A), nodule dry weight (B), total nitrogenase activity (C), specific nitrogenase activity (D), and nodule development (inset).
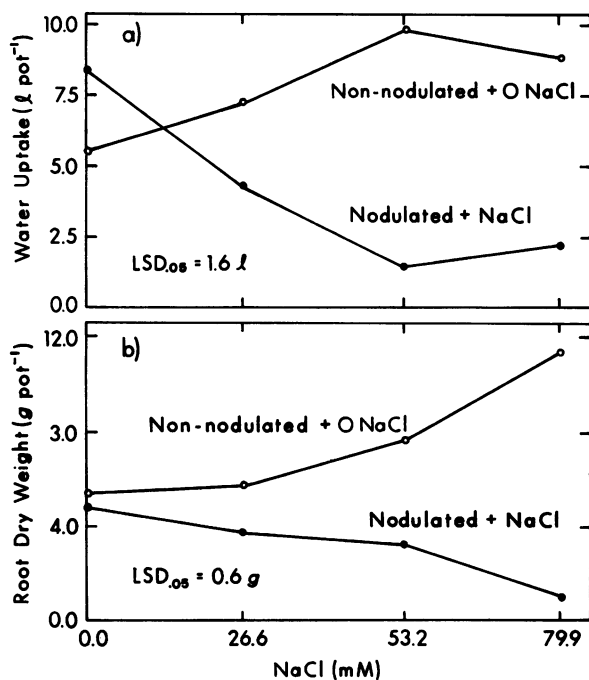

FIG. 3. Effect of NaCl to half-root systems on root weight and water uptake.

DISCUSSION

Nodule initiation in the legume-*Rhizobium* symbiosis involves a complex interaction between host root, rhizobial strain, and the environment. The processes of attachment and proliferation of rhizobia on root surfaces followed by infection thread formation in susceptible host root cells may be sensitive to the salinity. Since the supply of photosynthate required by the host for shoot growth, nodule initiation, development, and nodule function is also sensitive to salinity (11), evaluation of symbiotic processes exposed to stress requires that the processes be independently subjected to the stress. By utilizing a split-root growth system,

Table II. *Interaction between Time of Inoculation, Addition of NaCl to a Half-Root System and Nodule Formation by Soybean*

NaCl was added to a concentration of 53.2 mM.

Time between Inoculation and Salt Addition	Nodule No.[a]	Dry Wt		Shoot N	
		Nodule	Shoot		
h	no. pot^{-1}	g pot^{-1}		%	g N pot^{-1}
0	697	1.7	72.6	1.9	1.45
4	959	2.1	62.1	2.2	1.43
12	1167	2.6	59.0	2.5	1.52
96	1769	4.2	78.6	2.7	2.00
Control	2469	7.4	78.5	3.8	3.00

[a] All data are the means of two replications.

Table III. *Relative Salt Tolerance of Isolates from High Salt Treatments to Form Nodules in Saline Solutions*

Isolate from	Half-Root Exposed to	Nodule No.	Nodule Dry Wt	Nitrogenase Activity	
				1[a]	2[b]
mM NaCl		no. pot^{-1}	g pot^{-1}	μmol C$_2$H$_4$	
0.0	0.0	1229	2.97	176	65
79.9	0.0	974	2.55	271	108
0.0	79.9	47	0.25	6	57
79.9	79.9	10	0.18	2	59
LSD 0.05		411	0.76	34	

[a] Total nitrogenase activity, μmol C$_2$H$_4$ pot^{-1} h^{-1}.

[b] Specific nitrogenase activity, μmol C$_2$H$_4$ (g nodule)$^{-1}$ h^{-1}.

we were able to independently subject the nodulation process of the soybean-*R. japonicum* symbiosis to salinity and eliminate shoot stress as a variable in nodule initiation. Shoots of plants with their roots split between normal and salinized mediums have similar yield potential, leaf water potential, and stomatal conductance as plants with all their roots in a normal rooting medium (5, 12, 20).

The reduction in nodule initiation caused by NaCl salinity was not related to the survival of strain USDA 110 within the range of NaCl concentrations used in this experiment (Table I). This agrees with results reported by Carr and Ballard (8) and Singleton *et al.* (17) who reported that many *Rhizobium* could survive salt solutions approaching the concentration of sea water. Tu (22) claimed that rhizobial colonization of soybean roots was a limiting factor in nodule formation. Our study shows that nodule initiation is adversely affected by NaCl concentrations which are not inhibitory to rhizobial survival nor colonization of root surfaces.

Introducing NaCl into the rooting medium prior to inoculation reduced nodule formation at every concentration applied (Fig. 2). Total shoot N was correlated with the concentration of N in the shoot rather than with shoot dry weight (Fig. 1). This result is explained by the relationship between shoot yield potential of plants with half their roots salinized and the effect of salinity directly on the nodule formation process. Singleton and Bohlool (20) demonstrated that leaf expansion rates and dry matter yield potential of partially salinized soybean plants were comparable to nonsalinized controls. Salinity in the rooting environment at inoculation reduced nodule number and mass to such an extent in this experiment that there was inadequate N$_2$-fixation to meet the N requirements of the unstressed shoot. Similar dilution of N in the shoot of corn plants with roots under partial osmotic stress was reported by Bingham and Garber (5) and is also in evidence in Table II. Shoot N concentrations of legumes with the whole root system salinized do not decline with

respect to controls as osmotic stress is increased (3, 9, 20). When the whole plant system is stressed, shoot growth (the sink for fixation products) is limited and more in balance with the salinity related reduction in nodulation than is the case in our experiment.

Our experiment shows that the process of nodule initiation in soybean is extremely sensitive to NaCl. A reduction in nodulation of 50% compared to maximum nodule number and mass occurred with only 26.6 mM NaCl in the rooting medium (Fig. 2). Tu (22) found that up to 102 mM NaCl in the rooting medium of soybean did not result in a decline in nodule number; however, inoculation of the rooting medium with *R. japonicum* was performed prior to the institution of salinity treatments. Sensitive steps in the nodule initiation process may have already been completed by the time roots were exposed to salt.

Table II emphasizes the sensitivity of the early events in the nodule formation process. Nodule number and mass and N accumulation were increased by delaying the time between inoculation and the addition of 53.2 mM NaCl. Nodule number and mass were not as affected by 53.2 mM NaCl compared to the first experiment (Fig. 2). This difference can be explained by two factors. First, the salinity treatments were instituted 2 h before inoculation in the first experiment and at inoculation for the earliest treatment in this experiment. Second, the first experiment was conducted in the glasshouse during December-January while this experiment was planted in August. The greater yield potential in this experiment is in evidence by comparing the N accumulation by controls in Figure 1 and Table II.

The development of nodule tissue following infection is more resistant to salinity (Fig. 2, inset), Nodule size at 79.9 mM NaCl was 50% of the no-salt control while nodule number was less than 10% of the control. it has been shown that reduced numbers of nodules on soybean roots is compensated for by an increase in the average weight of a nodule so that total nodule weight remains approximately constant as the number of nodules declines (19). Apparently, NaCl stress limits this compensatory response so total nodule mass declined with nodule number.

Total nitrogenase activity followed the reduction in nodule number and mass; however, the nitrogenase system was more tolerant of exposure to NaCl since nodule specific activity was not affected except at the highest level of salinity (Fig. 2). This is consistent with the results of Singleton and Bohlool, (2) which showed that nodule function was relatively more resistant to salt stress than was plant growth.

Data for root weight and water uptake in the split-root system explain how plants with one-half the root system exposed to salt can have shoot growth similar to the nonsalinzed control (Fig. 3). Reduced root growth and water uptake by salinized half-root systems was compensated by root growth and water uptake by the nonsalinized side. Leaf osmotic potential and leaf expansion rates of split-root soybean plants are relatively unaffected when even 120 mM NaCl is applied to one-half the root system of soybean (20).

Although root proliferation by the salinized half-root systems were affected (Fig. 3), this would not reduce available nodulation sites and explain the observed effects of salinity on nodule formation. Singleton (18) demonstrated that soybean root systems supported the same number of nodules whether half or whole root systems were inoculated. Data from the competition experiments of Kosslak *et al.* (13) also indicated that the events within hours of inoculating soybean root radicles determine the number of nodules formed during later growth by competing strains of *R. japonicum*. These results imply that there are many times more available nodule sites than required for plant growth.

Some workers have tested a number of rhizobial strains to determine if strain selection could increase nitrogen fixation in saline environments (4, 15). We made isolates from nodules formed in the 79.9 mM NaCl treatment of the first experiment to determine whether these isolates were variants of the original culture and capable of increased nodule formation under saline conditions. Although the isolates from the salinity treatment formed more nodules in 79.9 mM NaCl (Table III) than isolates made from the nonsalinized treatment, the difference was small and nitrogenase activity was not appreciably enhanced. Isolates from the nonsalinized control produced more nodules and greater nodule weight in the 0.0 mM NaCl treatment of this experiment yet had substantially reduced acetylene reduction activity than isolates from 79.9 mM NaCl. The isolates made from the first experiment had variable symbiotic properties; this did not include, however, an increased ability to form nodules in saline culture medium.

In conclusion, the early processes involved in nodule formation by soybean were extremely sensitive to NaCl. Even low concentrations (26.6 mM NaCl) caused significant reductions in nodule number and weight. As a result, shoot nitrogen yield was limited by insufficient nodule tissue to meet the N requirements of relatively unstressed shoots. The split-root technique employed in these experiments allowed the nodule formation process to be independently subjected to salinity stress. Since shoots remained relatively unstressed, reduced nodulation observed on salinized half-root systems was not confounded with salinity related reduction in shoot growth potential (the sink for nodule products). Nodule development as indicated by the average weight of a nodule and nodule function (specific nitrogenase activity) were relatively less sensitive to salt than nodule initiation. The ability of *R. japonicum* strain USDA 110 to survive and colonize root surfaces was not affected by salinity. The use of isolates made from the high salt treatment as inoculum in a saline environment indicates that nodulation failure was due primarily to the effects of salinity on plant root infection sites.

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